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WESTERN IMMUNOBLOT ANALYSIS OF TWENTY-ONE SNAKE VENOMS FROM THREE SNAKE FAMILIES

by

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*Summer Research Student

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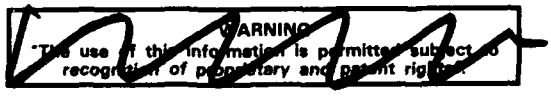

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ABSTRACT

Snake venom components antigenically similar to those found in *Vipera russelli* venom was investigated across a wide spectrum of species. A polyvalent antiserum against *Vipera russelli* venom was raised in rabbits, and used in Western immunoblotting to probe nitrocellulose filters that held SDS-PAGE-size-separated snake venom proteins from twenty one different species, ranging from other *Vipera spp.* to *Naja spp.* It was found that all species, even those distantly related to *Vipera russelli*, contained at least one immunologically cross-reacting component. The elapid samples were found to contain some of the strongest cross- reactive components, while many of the more closely related snakes showed weaker cross-reactivity. There appeared to be no correlation between the intensity and number of cross-reactive bands, and the relative evolutionary distance from *Vipera russelli*.

INTRODUCTION

Snake venom is known to be a complex mixture of organic and inorganic compounds (4, 6, 18). It is mainly the protein components of the venom which display pharmacological activity, are responsible for the damaging and lethal effects associated with snake bites, and are immunogenic (5, 11). Unlike immunoelectrophoresis and immunodiffusion, Western immunoblotting allows relatively easy determination of the size and number of protein subunits in a complex mixture that will bind specific antibodies (7). With this method, several studies, using antibodies raised against a single purified snake venom protein, have been performed to determine the number of antigenically cross-reactive proteins in venoms from various snake species. Lomonte *et al.*, (14), using antibodies against *Bothrops asper* myotoxin, detected cross-reactive proteins in *Bothrops nummifer, B. godmani, B. picadoi,* and *Agkistordon bilineatus* that had the same molecular weight (16,000) in all species except *B. picadoi,* which had a molecular weight of 24,000. Using monoclonal antibodies against Mojave toxin from *Crotalus scutulatus scutulatus,* Rael *et al.*, (19) found that there was cross-reactivity with proteins in *Crotalus basciliscus, C. durissus durissus, C. d. terrificus, C. horridis horridis,* and *C. viridis concolor* venoms. Arumae *et al*, (2) raised monoclonal antibodies against Vipera lebetina nerve growth factor and detected crossreactive proteins in *Vipera ursini, V. berus berus, Echis carinatus, Bungarus caeruleus, Agkistrodon halys, Naja naja atra, Naja naja oxiana, Naja naja*, and the mouse salivary gland. Similar experiments have been performed by other researchers (9, 26).

While these studies have been important in defining cross-reactive proteins using antibodies against a single specific protein

from one species of snake, there has been no corresponding work done on determining the number of cross reactive proteins using a polyvalent antiserum raised against the total venom from one species of snake. Since commercial antivenins are merely polyvalent antisera, it would be useful to determine the number of proteins in various snake venoms that this antiserum will react with. To this end, twenty-one different snake venoms (representing three different subfamilies) were Western blotted and allowed to react with polyvalent *Vipera russelli* antiserum. It was found that there were a great number of cross-reactive protein epitopes in many of the venoms studied.

MATERIALS AND METHODS

Snake Venoms

All snake venoms used in this study were obtained from the Sigma Chemical Co. (St. Louis, MO., USA) in lyophilized powder form.

Vipera russelli Antivenin

Vipera russelli venom (2 mg/ml in phosphate buffered saline PH7.4) was added to an equal volume of 2% bentonite suspension, mixed and incubated for one hour at 37°C. One ml of the bentonite-adsorbed venom was injected into New Zealand albino rabbits subcutaneously and intramuscularly at several sites. Two booster injections of 0.10 ml bentonite-adsorbed venom were given at weekly intervals. The rabbits were then bled and the gamma globulin portion of the serum was precipitated using 45% ammonium sulfate. The specific antibody activity was measured by an indirect enzyme-linked-immunosorbent assay.

Western Blotting Analysis

Snake venoms were reduced and size separated by the method of Mandrell and Zollinger (15). Samples were reduced with 1% 2-mercaptoethanol and 1% sodium dodecyl sulfate (SDS) and electrophoresed in 12.5% polyacrylamide gels containing 0.1% Empigen BB (alkyldimethylbetaine; Albright and Wilson, UK) detergent. This zwitterionic detergent was used because the presence of SDS in the gels appeared to cause denaturation of the immunogenic sites in the snake venom samples.

Polypeptides, resolved by electrophoresis, were transferred to nitrorocellulose (Bio-Rad Laboratories, Richmond) in a Bio-Rad Transblotter equipped with a Bio-Rad 160/1.6 power supply, using 25 volts for 16-18 hrs at 4°C. The electroblot buffer consisted of 0.025 M Tris-0.192 M glycine, 20% methanol and 0.05% Empigen BB. The nitrocellulose was then probed with the antiserum using a slight modification of the method of Burnette (7). The filter was washed with 0.1% bovine serum albumin (BSA) in 0.9% NaCl-10mM Tris (TS), pH 7.4 for 30 min to block non-specific binding of the antibodies. Then, 0.2 ml of *Vipera russelli* antiserum (1.0 mg/ml) was added and gently mixed for 90 min. The filters were then washed with TS containing 2% Nonidet P-40 (octylphenoxypolyethoxyethanol; Shell Oil Co., obtained through Sigma Chemical Co.) for 10 min and then TS for 10 min. A 1:1000 dilution of goat-anti-rabbit IgG alkaline phosphatase conjugated antibodies (Sigma Chemical Co.) was then added and gently mixed for 30 min. The wash procedure was then repeated.

Bands on the filter were visualized by the method of Bhatti and Done (3). A 10 mM Tris-5mM $MgSO_4$ solution containing 0.02% calcium-a-naphthyl-phosphate (Sigma Chemical Co.) and 0.02% Azoene Fast Violet B was added to the filters and shaken at 40°C until the dark brown

bands were clearly visible, at which point the filters were photographed.

RESULTS

Optimization of the Western Blot Protocol

Originally, the polyacrylamide gel electrophoresis was carried out by the method of Laemmli (12), and the Western blot by the method of Burnette (7). Even though separation of the polypeptide and transfer to nitrocellulose was effective with these methods, there was no antigens were detected (data not shown). It was found that there were two processes which caused this loss of detection. As in the protocol of Mandrell and Zollinger (15), the use of a zwitterionic detergent instead of SDS allowed efficient detection of antigens (data not shown). As well, it was found that the 5% BSA for blocking non-specific binding originally called for in the Burnette (7) method was masking the antigenic sites on the transferred polypeptides (data not shown). This effect was remedied by changing the BSA concentration to 0.1%.

Western Analysis of the Venoms

Equal amounts (100 µg) of snake venom proteins were reduced to polypeptides, electrophoresed, transferred to nitrocellulose and probed with the *Vipera russelli* antiserum. Samples of *Vipera russelli* venom showed six easily detectable bands and two faint bands, ranging from 120 to less than 20 kilodaltons (Figures 1A, 1B and 1C). Other *Vipera* species (*V. lebetina, V. palestinae, V. ammodytes*) showed a small number of moderately dark bands, all of which were of different molecular weight than the bands found in

Vipera russelli (Figure 1A). However, samples of *Naja spp.*, which are more distantly related from the *Vipera spp.*, indicated strongly reacting bands which have the same molecular weight as many of the bands in the *Vipera russelli* sample (Figure 1A). It was found that all of the venoms tested had at least one cross-reactive band, with most of the detected polypeptides being of a different molecular weight than of those detected in the *Vipera russelli* sample. There appeared to be one band, of 21-22 kilodaltons, that was found in all of the tested samples, suggesting that at least one antigenic polypeptide is conserved across all the species of snakes analyzed.

A large immunogenic smear was detected in several samples that corresponded to a molecular weight of less than 20 kilodaltons. This smear may be a result of the presence of the zwitterionic detergent in the polyacrylamide gels, or (more likely) represents a group of small molecular weight polypeptides which were not separated in the gel length used.

DISCUSSION

The results of this study address a completely neglected area in the cross-reactive analysis of snake venom proteins. Previously, only immunoelectrophoresis and immunodiffusion assays have been performed with complete polyvalent antisera to determine if there is cross-reactivity with snake venoms other than the one the antivenin was raised against (8, 13, 24, 25). These experiments have the disadvantage of only demonstrating that there is cross-reactivity, and do not determine the number of cross-reactive epitopes present. Recently, more specific immunoblot studies have been performed to define the number of cross-reactive epitopes similar to a single isolated snake

venom protein (2, 9, 14, 17, 19, 26).

After modifying the polyacrylamide gel electrophoresis/Western immunoblot protocol to obtain optimal results, we found that there was a great deal of cross-reactivity within the proteins from the venoms of snakes even distantly related to *Vipera russelli* when a polyvalent antiserum was used. The fact that most of the cross-reactive polypeptides were of a different molecular weight than that of the detected *Vipera russelli* polypeptides suggests that the cross-reactive epitopes are contained in polypeptides of different size, and most probably of some evolutionary distance from *Vipera russelli* (21, 22).

While some researchers place emphasis on the presence of cross reactive proteins as an indication of the phylogeny of snakes (8, 26), there are studies that make such conclusions dubious. It has been found that the components within the venom of a single individual snake may vary due to age or the seasons (10, 16). Two studies have demonstrated that *Echis carinatus* venom components vary from individual to individual (20, 23). It was also shown that snakes of the same species, but from different geographical areas, will have marked differences in the composition of their venoms (1, 17). Thus, the cross-reactive component detected may not always be present in any given sample. When one considers the results obtained in this study, there is further evidence against using the number of cross-reactive epitopes and the strength of their detection to formulate snake phylogeny. As can be seen in Figures 1A-C, *Vipera russelli* has a stronger cross-reaction with all the *Naja* species than with any of the other *Vipera* species. If cross-reactivity was a direct indication of evolutionary distance, one would expect to see the strongest cross-reactivity in the other *Vipera* species, followed by the

Bitis, *Cerastes*, and *Echis* species, as these are known to be more to be more closely related to *Vipera russelli* than the *Naja* species. Since there is actually stronger cross-reactivity in the elapid samples, it is clearly impossible to construct an accurate phylogeny using the presence of cross-reactive epitopes. It can be concluded, however, that there is a great deal of conservation of immunogenic epitopes, even across totally unrelated species.

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FIGURE 1A

WESTERN IMMUNOBLOT OF SELECTED SNAKE VENOM PROTEINS

100 µg of each snake venom was size separated on a 12.5% polyacrylamide gel and transferred to nitrocellulose. The filter was probed with a rabbit-anti-*Vipera russelli* antiserum, and detected with goat-anti-rabbit IgG conjugated to alkaline phosphatase (see Materials and Methods for further detail). (1) *Vipera russelli* (Russell's Viper) venom; (2) *Vipera lebetina* (Leventine Viper) venom; (3) *Vipera palestinae* (Palestinian Viper) venom; (4) *Vipera ammadoytes* (Horn Viper) venom; (5) *Naja haje* (Egyptian Cobra) venom; (6) *Naja naja* 4378 (Indian Cobra) venom; (7) *Naja naja* 9125 venom; (8) *Naja melanoleuca* (Black-lipped Cobra) venom and (9) SDS molecular weight markers.

FIGURE 1B

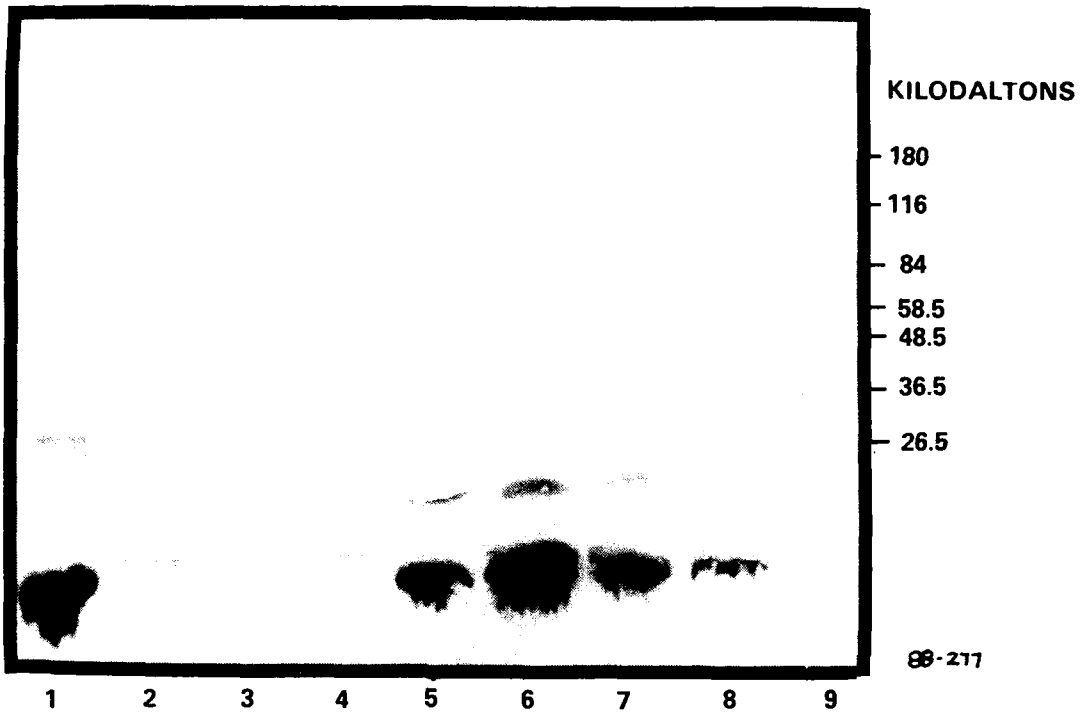
WESTERN IMMUNOBLOT OF SELECTED SNAKE VENOM PROTEINS

Samples were analyzed as outlined in Figure 1A. (1) *Vipera russelli* venom; (2) *Bothrops nummifer* (Juping Pit Viper) venom; (3) *Bothrops lansbergii* (Lansberg's Viper) venom; (4) *Bothrops jararacae* (Jararaca) venom; (5) *Cerastes cerastes* (Mojave Desert Sidewinder) venom; (6) *Crotalus viridis oregansis*- (Northern Pacific Rattlesnake) venom; (7) *Crotalus molassus molassus* (Northern Black-Tailed Rattlesnake) venom; (8) Atheris *squamigera* (Green Bush Viper) and (9) SDS molecular weight markers.

FIGURE 1C

WESTERN INNUMOBLOT OF SELECTED SNAKE VENOM PROTEINS

Samples were analyzed as outlined in Figure 1A. (1) *Vipera russelli* venom; (2) *Agkistrodon piscivorus* (Eastern Cottonmouth) venom; (3) *Agkistrodon rhodostoma* (Malayan Pit Viper) venom; (4) *Bitis gabonica* (Gaboon Viper) venom; (5) *Crotalus basciliscus* (Mexican West-Coast Rattlesnake) venom; (6) *Echis carinatus* (Saw-Scaled Viper) venom; (7) *Crotalus viridis viridis* (Prairie Rattlesnake) venom and (8) SDS molecular weight markers.



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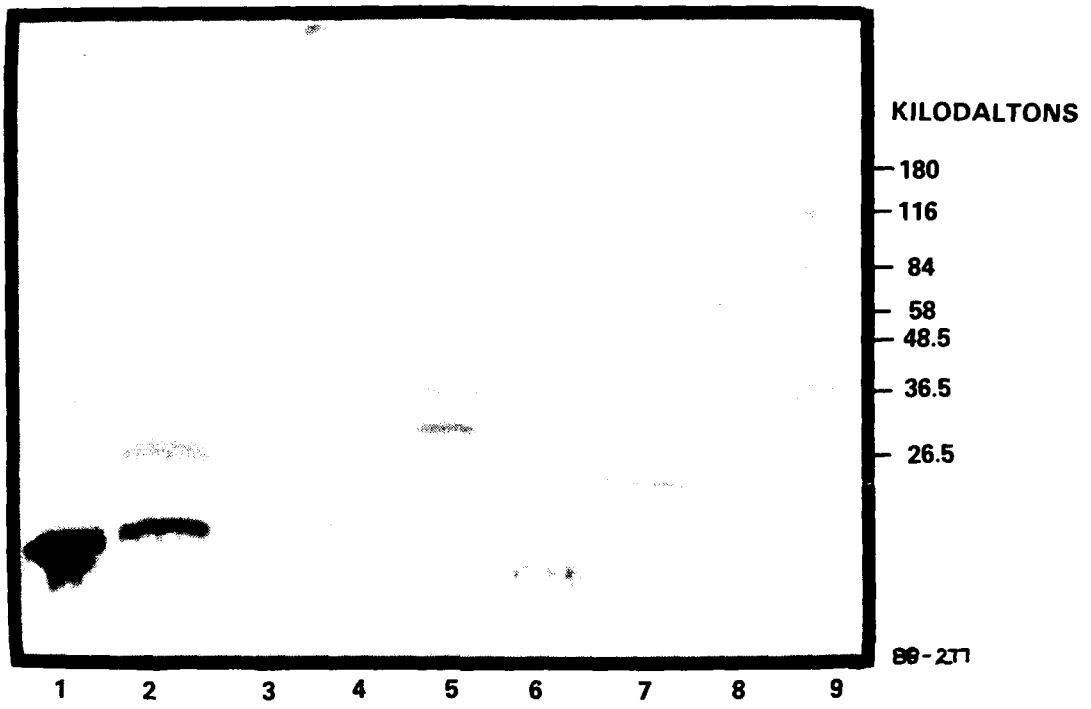

Figure 1B

WESTERN IMMUNOBLOT OF SELECTED SNAKE VENOM PROTEINS

Samples were analyzed in Figure 1A. (1) *Vipera russelli* **venom; (2)** *Bothrops nummifer* **(Juping Pit Viper) venom; (3)** *Bothrops lansbergii* **(Lansberg's viper) venom; (4)** *Bothrops jararacae* **(Jararaca) venom; (5)** *Cerastes cerastes* **(Mojave Desert Sidewinder) venom; (6)** *Crotalus viridis oregansis* **(Northern Pacific Rattlesnake) venom; (7)** *Crotalus molassus molassus* **(Northern Black-Tailed Rattlesnake) vemom; (8)** *Atheris squamigera* **(Green Bush Viper) and (9) SDS molecular weight markers.**

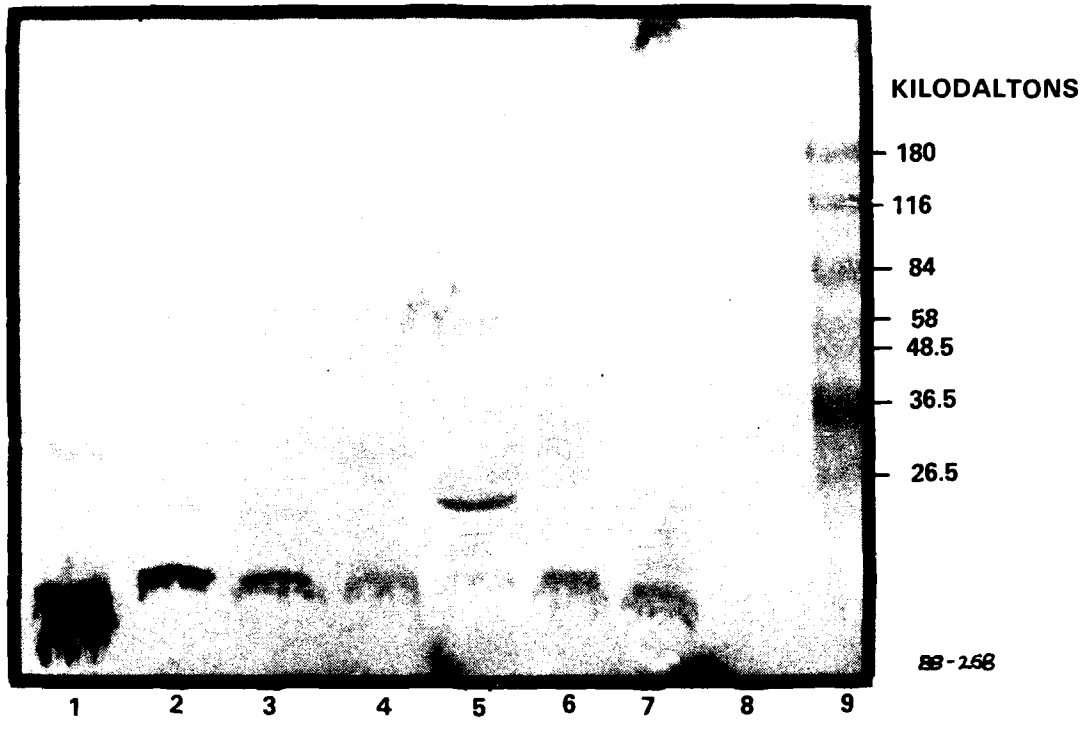

Figure 1C

WESTERN IMMUMOBLOT OF SELECTED SNAKE VENOM PROTEINS

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ABSTRACT

Snake venom components antigenically similar to those found in Vipera russelli venom was investigated across a wide spectrum of species. A polyvalent antiserum against Vipera russelli venom was raised in rabbits, and used in Western immunoblotting to probe nitrocellulose filters that held SDS-PAGE-size-separated snake venom proteins from twenty one different species, ranging from other Vipera spp. to Naja spp. It was found that all species, even those distantly related to Vipera russelli, contained at least one immunologically cross-reacting component. The elapid samples were found to contain some of the strongest cross- reactive components, while many of the more closely related snakes showed weaker cross-reactivity. There appeared to be no correlation between the intensity and number of cross-reactive bands, and the relative evolutionary distance from Vipera russelli.

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